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(54) Title: BIOSPECIFIC ANTIBODY TO CANCER CELL AND ENZYME WITH PRODRUG-ACTIVATING CHARACTERISTICS

(57) Abstract

The present invention provides a hybrid monoclonal antibody having specificities against a human cancer cell and a prodrug-activating enzyme, a polydoma which produces said antibody and an anti-human-cancer-protein complex comprising said antibody and prodrug activating enzyme which is immunologically coupled thereto, and methods of using said antibody in combination with anticancer prodrug for therapy of cancer.

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DESCRIPTION

BIOSPECIFIC ANTIBODY TO CANCER CELL AND ENZYME WITH PRODRUG-ACTIVATING CHARACTERISTICS.

The present invention relates to a bispecific antibody-enzyme complex that serves well as an anticancer therapeutic drug. More specifically, the present invention relates to a hybrid monoclonal antibody (hereinafter also referred to as hybrid MoAb) wherein one of the two specificities is to human cancer cells and the other is to a prodrug-activating enzyme, and a polydoma that produces said antibody.

The present invention also relates to an anti-human-cancer-protein complex obtained by immunologically binding the above-mentioned enzyme to the above-mentioned hybrid MoAb.

Many investigations have been made of what are called "antibody missile therapy drugs", antitumor immunocomplexes prepared by binding an antitumor antibody to a chemotherapeutic agent or a biotoxin which aims at selective destruction of cancer cells; some successes have been achieved in blood-related cancers such as leukemia and lymphoma. However, no satisfactory results have been obtained in actual clinical application. Particularly with respect to solid cancers, much remains unsolved, including the problem of serious side effects. This is mainly because 1) it is impossible to introduce a sufficient amount of anticancer agent into cancer cells due to the limited number of tumor-related antigens present on the cancer cell surfaces, 2) severe side effects hamper clinical application of highly cytotoxic biotoxins (e.g., ricin, *Pseudomonas aeruginosa* exotoxin) to obtain a lethal effect on cancer cells in the presence of so few tumor-related antigens, and 3) cancer cells without target antigens are capable of proliferation uninfluenced by the cytotoxic action of missile therapeutic drugs, since human cancer cells are generally highly diverse and there is almost no possibility that all have the same kind of tumor-related antigen. It is particularly difficult to develop a therapeutic method that overcomes the diversity of cancer cells. Proposed methods include therapy using numerous kinds of anticancer antibody

(antibody cocktail therapy), but this therapy is unrealistic, since it is very difficult to prepare numerous kinds of cancer-specific antibody.

SUMMARY OF THE INVENTION

With the aim of solving the above-mentioned problems with conventional anticancer missile therapeutic drugs, the present inventors investigated a recently developed bispecific antibody and developed the present invention. Accordingly, the inventors prepared a bispecific antibody capable of binding to both an enzyme that converts an inactive anticancer prodrug into active type and to a human cancer cell, and administered an immunocomplex comprising said antibody and said enzyme, as well as an inactive prodrug, to cancer patients, thus developing an anti-human-cancer-protein complex exibiting a cytotoxic effect selectively on cancer cells, regardless of their diversity.

The prodrug itself is present in blood and other organs and tissues in an inactive form; only in the vicinity of the target cancer tissue is the prodrug decomposed and activated by the anti-human-cancer-protein complex of the present invention, as bound to human cancer cells, to exhibit its anticancer activity. Thus, the prodrug has almost no side effects in the administration method using the anti-human-cancer-protein complex of the present invention. Administration using the anti-human-cancer-protein complex of the present invention is also characterized in that anticancer activities are exhibited against cancer cells which are present in the vicinity of the target cancer tissue but are free of target antigens, cytotoxic effect being exhibited regardless of the diversity of cancer cells. Accordingly, one object of the present invention is to provide a bispecific hybrid monoclonal antibody wherein one of the two specificities is to human cancer cells and the other is to a prodrug-activating enzyme, and a polydoma that produces said antibody.

Another object of the present invention is to provide an anti-human-cancer-protein complex comprising the bispecific hybrid MoAb described above and a prodrug-activating enzyme immunologically coupled thereto.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the cytotoxicity of a tripeptidated drug (Boc-Gly-Gly-Arg-ADR; - -) and its activated body (ADR; - ○ -) on gastric cancer cell line NUGC4 (see Example 4).

Fig. 2 shows the cytotoxicity of a tripeptidated drug (Boc-Pro-Gly-Arg-TAN-1120; - ● -) and its activated body (TAN-1120; - ○ -) on renal cancer cell line AM-RC-6 (see Example 4).

Fig. 3 shows the cytotoxicity of a tripeptidated drug (Boc-Gly-Gly-Arg-PM; - ● -) and its activated body (PM; - ○ -) on renal cancer cell line AM-RC-6 (see Example 4).

Fig. 4 shows a chromatographic pattern of the trypsin-hydrolyzed product of Boc-Gly-Gly-Arg-ADR (see Example 6).

Fig. 5 shows a chromatographic pattern of the UK-hydrolyzed product of Boc-Gly-Gly-Arg-PM (see Example 6).

Fig. 6 shows the antibody dilution curve of the culture supernatant of anti-hTfR-anti-UK bispecific antibody producing mouse tetraoma UTF 20-7. (see Example 9)

DETAILED DESCRIPTION OF THE INVENTION

The above-mentioned polydoma that produces a bispecific hybrid MoAb is prepared, for example, by fusing a hybridoma that produces an anti-human-cancer antibody with another hybridoma that produces an antibody against a prodrug-activating enzyme. Any anti-human-cancer-antibody-producing hybridoma can serve for this purpose, as long as it produces an antibody capable of specifically binding to human cancer cells. Examples of such hybridomas include mouse hybridoma 22C6 [IFO 50172, FERM BP-2054] [cf. Japanese Unexamined Patent Publication No. 79970/1990], which produces an MoAb against human transferrin receptor (hereinafter also referred to as hTfR), and mouse hybridoma RCS-1 [IFO 50184, FERM BP-2333] [cf. Japanese Patent Application No. 62939/1989], which produces an MoAb against human renal cancer. Representative examples of target antigens for the anti-human-cancer antibodies produced by these hybridomas, i.e., target antigens for cancer cells to which the bispecific hybrid MoAb of the present invention binds specifically, include cancer cell membrane surface antigens such as tumor-related antigens, immun competent cell surface receptors and virus-infected cell surface antigens. Of these, hTfR, a tumor-related antigen, is often used. Oth r

examples of target antigens include carcinoembryonic antigen (CEA), α-fetoprotein, some cancer-related sugar chain antigens such as CA19-9 [S. Hakomori: Cancer Research, *45*, 2405 (1985)], the B-cell lymphoma membrane immunoglobulin idio-type [R. A. Miller et al.: New England Journal of Medicine, *306*, 517 (1982)] and the T-cell lymphoma membrane immunoglobulin idio-type [L. L. Lanier et al.: Journal of Immunology, *137*, 2286 (1986)].

In preparing a hybridoma that produces an antibody against a prodrug-activating enzyme, an ordinary hybridoma preparation method is used [G. Köhler et al.: Nature, *256*, 495 (1975)]. For example, animals are immunized with the enzyme in accordance with a standard method, and the resulting antibody-producing cells are fused with myeloma cells etc.

Examples of immune animals include rabbits, rats, mice and guinea pigs, with preference given to mice in the case of MoAb preparation. Inoculation can be achieved by an ordinary method. For example, the mouse receives subcutaneous or intraperitoneal inoculation of the enzyme at the back or abdomen at a dose of 1 to 100 μg, preferably 10 to 25 μg, in emulsion in an equal volume (0.1 mℓ) of saline, in the presence of Freund's complete adjuvant, 3 to 6 times once every 2 to 3 weeks.

Of these immune animals, for example, mice, individuals with high antibody titer are selected. Three to five days after final immunization, spleens and/or lymph nodes are collected, and antibody-producing cells contained therein are fused with myeloma cells. Fusion can be achieved in accordance with a known method. Examples of fusogens include polyethylene glycol (hereinafter also referred to as PEG) and Sendai virus, with preference given to PEG. Example myeloma cell lines include NS-1, P3U1 and SP2/0, with preference given to P3U1. A preferred ratio of, for example, splenocytes and myeloma cells, is 1:1 to 10:1. It is recommended that this cell mixture be incubated at 20 to 37°C, preferably 30 to 37°C, in the presence of a PEG with a molecular weight of about 1,000 to 9,000 at a concentration of 10 to 80% for 3 to 10 minutes.

Various methods are available for screening the antibody-producing hybridomas described above. For example, human cancer cells or enzyme proteins are adsorbed to a microplate to prepare an antigen-sensitized plate, to which is added the culture supernatant of the hybridomas obtained by cell fusion. This is followed by determination of antibody titer in the culture

supernatant by enzym immunoassay (hereinafter also referred to as EIA) for detecti n of plate-bound specific antibody. Hybridomas positive for antibody activity are selected, cultured in HAT (hypoxanthine-aminopterin-thymidine) medium etc. and immediately subjected to cloning, which can be done easily by the limiting dilution method. The antibody titer of the culture supernatant of the cloned hybridomas is also determined by the EIA procedure described above; monoclonal hybridomas that stably produce a potent antibody can thus be selected and cultured. In this case, a hybridoma that produces a neutralizing antibody against a prodrug-activating enzyme, such as urokinase, can also be used as a parent cell in polydoma preparation.

There are several methods of preparing a polydoma that produces the bispecific hybrid MoAb of the present invention [e.g., Yoji Aramoto et al.: Proteins, Nucleic Acids and Enzymes, *33*, 217 (1988)]. All of them are suitable; examples are as follows: 1) The above-mentioned HAT-resistant hybridoma, that produces an antibody against a prodrug-activating enzyme, is acclimated step-by-step to a culture medium containing 5-bromodeoxyuridine (hereinafter also referred to as 5-BrdU), and a thymidine kinase-deficient strain is cloned to make it HAT-sensitive. Similarly, an HAT-resistant hybridoma that produces an anti-human-cancer specific antibody is made resistant to 8-azaguanine (hereinafter also referred to as 8-AZG), and a hypoxanthine-guanine-phosphoribosyl transferase-deficient strain is cloned to make it HAT-sensitive. Next, these two cloned strains are fused by a standard method to yield tetraomas, from which a tetraoma that secretes a hybrid MoAb capable of binding to both human cancer cells and a prodrug-activating enzyme is selected on HAT medium and cloned. 2) A hybridoma that produces an anti-human-cancer-cell specific antibody is labeled with fluorescein isothiocyanate (hereinafter also referred to as FITC), and another hybridoma that produces an antibody against a prodrug-activating enzyme is labeled with tetramethyl rhodamine isothiocyanate (hereinafter also referred to as TRITC), followed by fusion of these two in accordance with a standard method. The resulting cell suspension is applied to a fluorescence-activated cell sorter (hereinafter also referred to as FACS), and a tetraoma that shows both the green fluorescence of FITC and the red fluorescence of TRITC is selected and cloned. Also, it is possible to use the markers for the two parents in totally reverse combination to select and clon the desired tetraoma.

These procedures of cell fusion employ a fusogen such as Sendai virus or PEG, or a means such as electric stimulation. It is preferabl to use PEG. An example mode of PEG use is described below, but this is not to be construed as limitative. A PEG with a molecular weight of about 1,000 to 9,000 is used at a concentration of about 10 to 80%; treatment time is about 0.5 to 30 minutes. As a preferred mode of use, about 35 to 55% PEG 6,000 is kept in contact with cells at 37°C for about 4 to 10 minutes to achieve efficient fusion.

Polydoma selection can be carried out using the HAT medium described above and other means. For this purpose, 8-AZG, 6-thioguanine (6-TG) or 5-BrdU is used for drug acclimation to obtain corresponding drug-resistant strains. Also, various selection media are used to introduce a new marker into fused cells. Examples of such selection media include media supplemented with neomycin or hygromycin B [B. Sugden et al.: Molecular and Cellular Biology, *5*, 410 (1985)].

As stated above, it is also possible to use a method in which hybridomas labeled with different fluorescent pigments are fused, followed by sorting of a double-labeled hybrid hybridoma by means of FACS [L. Karawajew et al.: Journal of Immunological Methods, *96*, 265 (1987)].

Various methods are available for screening hybrid antibody-producing polydomas, including combinations of the following methods and their modifications: (1) the method employing two kinds of EIA techniques using the above-mentioned antigen-sensitized plate to which human cancer cells or enzyme have been adsorbed, (2) the EIA method in which the subject culture supernatant is added to a microplate to which human cancer cells are adhered, followed by addition of a prodrug-activating enzyme labeled with horseradish peroxidase (hereinafter also referred to as HRP) and detection of bispecific antibody, and, when using an antibody against a prodrug-activating enzyme belonging to a subclass different from that of the anti-human-cancer specific antibody, (3) the EIA method, in which the subject culture supernatant is added to a microplate to which human cancer cells are adhered, followed by addition of an HRP-labeled specific antibody against the mouse IgG subclass and detection of bispecific antibody.

The polydoma positive for bispecific antibody activity is immediately subjected to cloning, easily be achieved by th limiting dilution method etc. The antibody titer of the culture supernatant of the clon d polydoma is

determined by the method described above, and a polydoma that stably produces a potent antibody is selected, whereby the desired polydoma (e.g., mouse tetraoma UTF20-7(IFO 50260, FERM BP-3156) obtained in following Example, or other methods) that produces the monoclonal bispecific antibody can be obtained.

The polydoma of the present invention described above can be cultivated normally in liquid medium, or in the abdominal cavity of animals (e.g., mammalians such as mice) by a known method. Purification of the antibody in the culture broth or ascites can be achieved using a combination of known biochemical techniques. For example, the cell culture broth or ascites fluid is centrifuged, and the resulting supernatant separated and subjected to salting-out (normally with ammonium sulfate or sodium sulfate). The resulting protein precipitate is dissolved in an appropriate solution, followed by dialysis. The solution is then subjected to column chromatography (using an ion exchange column, gel filtration column, protein A column, hydroxyapatite column etc.) to separate and purify the desired antibody. From each liter of culture supernatant, the separation and purification procedures described above yield about 1 to 10 mg of a bispecific MoAb of a purity not less than 90% by protein weight. Also, from 20 mℓ of ascites fluid, the same MoAb is obtained in an amount of about 2 to 20 mg.

The bispecific MoAb thus obtained is uniform as a protein and, for example, $F(ab')_2$ fragments etc. capable of binding to both human cancer cells and a prodrug-activating enzyme can be obtained, for example, by treatment with protease (e.g., pepsin). These fragments can be used for the same purpose as the bispecific MoAb of the present invention.

A tetraoma formed between a hybridoma that produces an anti-human-cancer-cell MoAb and another hybridoma that produces an antibody against a prodrug-activating enzyme is included in the polydoma that produces the hybrid MoAb of the present invention, but any trioma formed between a hybridoma that produces one MoAb and a cell that produces the other MoAb, or any hybridoma obtained by immortalizing two kinds of cells that produce respective MoAbs using Epstein-Barr virus or other means. and then fusing them, can be used for the same purpose as the above-mentioned tetraoma, as long as it produces the bispecific MoAb of the present invention.

When these polydomas produce mouse IgG MoAb, it is possible to prepare a mouse-human chimeric antibody by deriving a DNA that encodes a

variable or hypervariabl region containing the antigen recogniti n site for said bispecific hybrid MoAb and binding thereto a gene that encodes the constant region of human IgG, using a gene manipulation technique [Z. Steplewski et al.: Proceedings of National Academy of Science, *85*, 4852 (1988)]. Such a chimeric antibody serves well in administration to humans, due to its low antigenicity.

In anticancer therapy using the bispecific MoAb of the present invention or a selective anti-human-cancer-protein complex prepared from a prodrug-activating enzyme and said bispecific MoAb, several methods are available, including (1) the method in which the bispecific MoAb of the present invention is administered to the cancer patient, and after sufficient time has elapsed for it to bind to cancer tissues and cells, the enzyme and then the prodrug are administered, (2) the method in which the bispecific MoAb and the enzyme are administered simultaneously, followed by prodrug administration, and (3) the method in which the hybrid MoAb is reacted with the enzyme, and after separation of the unreacted portion of the enzyme, the resulting anti-human-cancer-protein complex is administered to the cancer patient, followed by prodrug administration.

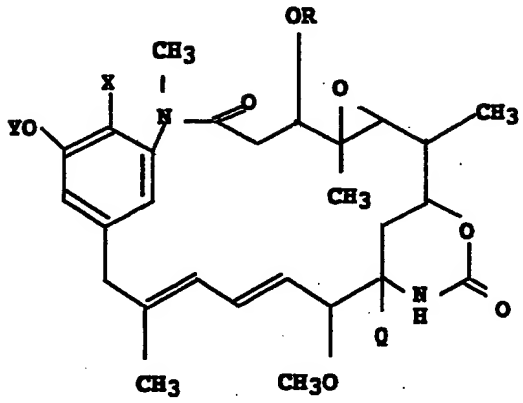
The bispecific MoAb or prodrug-activating enzyme of the present invention, or an anti-human-cancer-protein complex prepared therefrom, can be used for the treatment of various cancerous diseases in the form of a preparation such as an injection, with or without being formulated with an appropriate pharmacologically acceptable carrier, excipient, diluent or other additive, after germ removal by filtration using, for example, a membrane filter, as desired. Dose volume varies depending upon the type of target cancer, symptoms, route of administration and other aspects; but, for example, in intravenous administration to an adult human patient, it is normally about 0.02 to 1.0 mg/kg, preferably about 0.04 to 0.4 mg/kg, daily, as the bispecific antibody, or about 0.01 to 0.5 mg/kg daily, as the prodrug-activating enzyme.

Any prodrug-activating enzyme can serve for the present invention, as long as it shows prodrug-activating action, but it is preferable to use a protease (e.g., urokinase (UK), trypsin), which cleaves peptide bonds, or a glycosidase (e.g., glucuronidase), which cleaves sugar chain bonds. Of these enzymes, a protease, particularly urokinase, is preferred. Also, it is desirable that th enzym be a human-derived enzyme whose blood level is low or

which is not present in blood, and that the enzyme be produced by cancer cells [J. C. Kirchheimer et al.: Proceedings of National Academy f Science, USA, *86*, 5424 (1989)]. For example, in the case of urokinase, prodrugs comprising a drug active body and an appropriate peptide (e.g., Gly-Gly-Arg, Pro-Gly-Arg, Pyr-Gly-Arg) bound thereto can be used. In the case of glucuronidase, glucuronidated drugs can be used as prodrugs. Whether the prodrug is a peptidated drug or a glucuronidated drug, its toxicity is expected to be extremely lower than that of the original drug active body, or even nontoxic; therefore, it is capable of being activated in the vicinity of the cancer tissue and selectively destroying the cancer tissue when used in combination with an anti-human-cancer-protein complex comprising the bispecific antibody of the present invention and a prodrug-activating enzyme immunologically bound thereto.

Any anticancer agent can be used as the original drug for the above-mentioned prodrug, but preference is given to those in clinical application such as adriamycin, cisplatin, melphalan, methotrexate, mitomycin C, vincristine, puromycin and phenylenediamine mustard. Also, highly cytotoxic ansamitocins , TAN-1120 (represented by the following formula (II) wherein X represents OH) and related compounds may be used as anticancer agents. Examples of such compounds include compounds represented by the following formula (I) [cf. Japanese Patent Application No. 18560/1989, European Patent Publication No. 376176], their 4,5-deoxy bodies, and compounds represented by the following formula (II) [cf. Japanese Patent Application No. 178634/1989, European Patent Publication No. 376177] and their salts. In this case, any ansamitocin or TAN-1120 related compound can be used, as long as it possesses anticancer activity. In any case, as stated above, the drug is administered to the patient in the form of a nontoxic or weakly toxic prodrug, and is decomposed by the anti-human-cancer-protein complex of the present invention in the vicinity of the cancer tissue to exhibit its pharmacological activities.

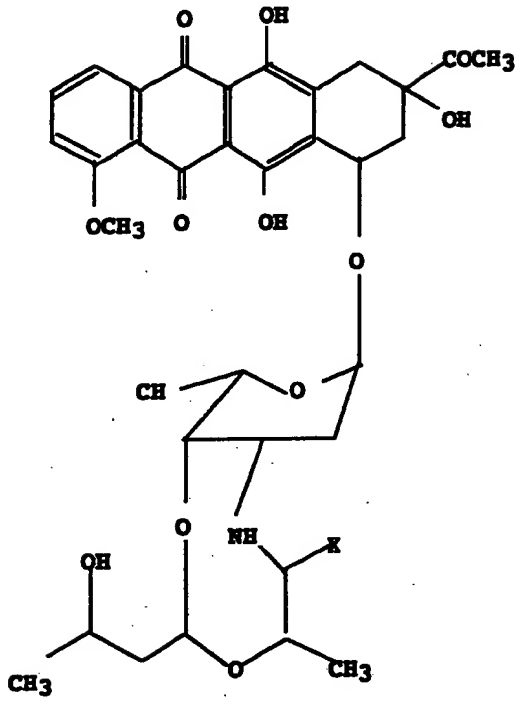
Ansamitocins includ compounds represented by the following formula:



(I)

wherein R represents a hydrogen atom or a carboxylic acid-derived acyl group; Q represents a hydroxyl group (OH) or a mercapto group (SH); X represents a chlorine atom or a hydrogen atom; Y represents a hydrogen atom, a lower alkylsulfonyl group, an alkyl group or an aralkyl group which may have a substituent.

TAN-1120 related compounds



(II)

wherein X represents a hydroxyl group or a hydrogen atom.

EXAMPLES

The present invention is hereinafter described in more detail by means of the following reference and working examples, but these are not to be construed as limitations on the scope of the invention.

The animal cell lines used in the reference and working examples are in deposition as listed in the table below.

Animal cell line	(IFO) IFO No.	(FRI) FERM No.
Mouse hybridoma UK 1-3	50176	BP-2083
Mouse hybridoma UK 1-87	50177	BP-2084
Mouse hybridoma UK 1-6	50208	BP-2548
Mouse hybridoma RCS-1	50184	BP-2333
Mouse hybridoma BG1-5	50219	BP-2688
Mouse hybridoma 22C6	50172	BP-2054
Mouse tetraoma UTF20-7	50260	BP-3156

IFO: Institute for Fermentation, Osaka

FRI: Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan

In the present specification, amino acids and peptides are represented by the abbreviation system adopted by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN). For example, the symbols given below are used. When there is a possibility that an optical isomer is present in a given amino acid, the symbol represents the L-body unless otherwise stated.

Gly: Glycine residue
Pro: Proline residue
Arg: Arginine residue
Pyr or PyroGlu: Pyroglutamic acid residue

Reference Example 1 Mixed hemagglutination (MHA)

Of the subject cells, adherent cells were dispensed to a 60-well microplate (produced by Nunc Intermed) at 500 cells per well and cultured for 24 to 48 hours. Non-adherent cells were suspended in a serum-free culture medium and dispensed to a plate at the same ratio as above, followed by centrifugation at 400 × g for 5 minutes to adsorb them to the plate, on the day of examination.

For preparing indicator blood cells, sheep red blood cells were washed three times with phosphate buffered saline (20mM bisodiumphosphate, 0.15M NaCl; pH7.5) (hereinafter also referred to as PBS) and suspended in PBS to obtain a 2% suspension. This suspension was mixed with an equal amount of mouse anti-sheep-red-blood-cell antibody (produced by Ortho Co.), diluted with PBS to a concentration 2.5 times the maximum agglutination value, followed by reaction at 37°C for 30 minutes. These blood cells were washed three times with PBS and resuspended at a concentration of 2%. Then, an equal amount of rabbit anti-mouse-IgG antibody (produced by Cappel Laboratories), diluted 25-fold with PBS, was added, followed by reaction at 37°C for 30 minutes. The reaction mixture was then washed three times with PBS and stored as a 2% suspension.

The cell-adsorbed plate was washed by sequential additions of a veronal-buffered saline (pH 7.4; hereinafter also referred to as VBS) containing 0.1 M $MgC\ell_2$-0.03 M $CaC\ell_2$-0.1% glucose and a solution containing 5% fetal calf serum (5% FCS-VBS). Subsequently, sample solution containing a mouse anti-human-cancer antibody was dispensed to each well, and the plate was kept standing at room temperature for 1 hour. After plate washing with VBS, indicator blood cells, in 0.2% dilution in 5% FCS-VBS,

were dispensed to ach well, and the plate was kept standing at room temperature for 40 minutes. Next, the unreacted blood cells were washed away with VBS, and the plate was observed microscopically. In the control test, in which no antibody was added, a rosette was found in not greater than 1% of the cells. A "positive" test was definded as a rosette formed by not less than 25% of the target cells.

Reference Example 2 Immunofluorescence (IF)

After cultivation, the subject cells were suspended in a 0.02% EDTA-PBS solution. This suspension was washed with a serum-free culture medium, and a solution containing a mouse anti-human-cancer antibody was added, followed by reaction at 4°C for 1 hour. After washing with culture medium, fluorescein-labeled anti-mouse-IgG antibody was added, followed by reaction at 4°C for 1 hour. After washing with PBS, the reaction product was observed using a fluorescent microscope.

Reference Example 3 Cell EIA using tumor cells

Target tumor cells were seeded to a Nunc Intermed 96-well microplate at 10,000 to 40,000 cells per well, followed by incubation in a carbon dioxide incubator at 37°C for 1 day. After culture supernatant removal, a solution containing a mouse anti-human-cancer antibody was added, followed by reaction at room temperature for 2 hours. The plate was then washed with a medium supplemented with 0.2% bovine serum albumin (hereinafter also referred to as BSA); a rabbit anti-mouse-IgG antibody labeled with horseradish peroxidase (hereinafter also referred to as HRP) was then added, followed by reaction at room temperature for 2 hours.

After plate washing, a 0.1 M citrate buffer containing ortho-phenylenediamine as the enzyme substrate and H_2O_2 was added to each well, followed by enzyme reaction at room temperature. After the reaction was terminated by the addition of 1 N sulfuric acid, the developed color was measured at a wavelength of 492 nm using Multiscan (produced by Flow Co.).

Reference Example 4 Preparation of hybridoma that produces anti-human-renal-cell-cancer monoclonal antibody

(1) Human renal cell cancer transplantation and serum immunization

A 2 × 2 mm tissu section was collected from a renal cancer patient's tumor tissue and transplanted subcutaneously to a nu/nu-BALB/c mouse. Upon subculture (normally 3 to 4 weeks after transplantation) of stable cell line AM-RC-3, serum was collected from the mouse recipient. A 0.5 mℓ portion of the serum was mixed and suspended in an equal amount of Freund's complete adjuvant. The resulting suspension was intraperitoneally administered to the same line of BALB/c mouse. Thereafter, the mouse was immunized with 0.5 mℓ serum from the above-mentioned recipient nude mouse at intervals of about 7 to 10 days. After a total of 7 immunizations, antibody titer was determined.

By the MHA method described in Reference Example 1, mice showing a high antibody titer against renal cancer cell line AM-RC-7 were subjected to the following experiment.

(2) Preparation of hybridoma

The immune mouse splenocytes obtained in (1) were fused with mouse myeloma cell line NS-1 by a standard method, followed by selection culture using HAT medium. The hybridomas grown were subjected to screening by the MHA method described in Reference Example 1, and the group of hybridomas showing high antibody titer were further cloned to yield the desired mouse hybridoma RCS-1 (FERM BP-2333, IFO 50184), which produces an anti-human-renal-cell-cancer MoAb. The RCS-1 antibody produced by the mouse hybridoma RCS-1 proved to belong to the IgG_1 subclass.

(3) Production of mouse MoAb

5×10^6 cells of mouse hybridoma RCS-1 were intraperitoneally administered to MCH(AF)-nu mice. About 4 weeks later, 5 to 10 mℓ of ascites fluid was collected. After salting-out with ammonium sulfate, the ascites fluid was purified using a DEAE-cellulose column. About 200 mg of purified mouse anti-human-renal-cell-cancer MoAb RCS-1 was obtained from 50 mℓ of ascites fluid.

(4) Properties of mouse MoAb

Reactivity of RCS-1 antibody against various tumor cell lines was determined by the methods described in Reference Examples 1, 2 and 3. RCS-

1 antibody was found to be positive for renal cancer cell lines AM-RC-3, AM-RC-6 and AM-RC-7, bladder cancer cell line T24 and lung cancer cell lines Luci-10 and PC-10, and negative for other cancers, namely gastric cancer, intestinal cancer, breast cancer and leukemia cancer cell lines; it was also negative for normal renal tissues.

Reference Example 5 EIA for anti-UK antibody assay

A 5 μg/mℓ UK solution was dispensed to a 96-well microplate at 100 μℓ per well. After the microplate was kept standing at 4°C overnight, 150 μℓ of PBS containing 2% casein and 0.01% thimerosal was added, to prepare a sensitized plate. After removing the added solution, the plate was washed with PBS containing 0.05% Tween 20 (hereinafter also referred to as PBS-Tw), and 100 μℓ of the subject mouse antibody solution was added, followed by reaction at room temperature for 2 hours. Similarly, after the plate was thoroughly washed with PBS-Tw, an HRP-labeled rabbit anti-mouse-IgG antibody was added, followed by reaction for 2 hours.

The procedure described in Reference Example 3 was then followed to determine the HRP activity bound to the solid phase.

Reference Example 6 EIA for anti-low-molecular-UK antibody assay

Using a low molecular UK (two chain-low molecular UK, supplied by JCR Co.) in place of the UK described in Reference Example 5, a plate sensitized with a low molecular UK was prepared, and anti-low-molecular-UK antibody titer was determined by the same method.

Reference Example 7 Fibrinolysis neutralization experiment

To a UK solution (final concentration 25 ng/mℓ), the subject anti-UK antibody solution was added, followed by reaction at 37°C for 1 hour. The reaction mixture was then injected to a fibrin agarose plate at 5 μℓ per well. After incubation at 37°C for 2 to 6 hours, fibrinolysis plaques (diameter) were measured to calculate the neutralizing capability of the anti-UK MoAb on UK enzyme activity.

Reference Example 8 Preparation of hybridoma that produces mouse anti-UK monoclonal antibody

(1) Immunization

To a 200 μg/mℓ solution of a commercially available UK (produced by Nippon Seiyaku) in saline, an equal amount of Freund's complete adjuvant was added; this mixture was thoroughly emulsified. The resulting emulsion was intraperitoneally and subcutaneously (at the back) administered to BALB/c mice (female, 20 μg/0.2 mℓ/mouse), followed by booster immunization at intervals of 2 to 3 weeks. The animals showing maximum serum antibody titer at 10 days after the third booster immunization received intravenous administration of a UK antigen solution (50 μg/0.1 mℓ saline/mouse).

(2) Cell fusion

Spleens were excised 3 days after final immunization, and a splenocyte suspension was prepared by a standard method (about 10^8 cells). Then, 2×10^7 mouse myeloma cells (P3U1) were added, and cell fusion was carried out using PEG 6000 by the method of Köhler and Milstein [Nature, *256*, 495 (1975)].

After completion of fusion, the cell mixture was suspended in what is called HAT medium, containing hypoxanthine, aminopterin and thymidine, followed by cultivation for 10 days. Immediately after parent cell selection, the HAT medium was replaced with HT medium of the same composition as HAT medium but lacking aminopterin, and cultivation was continued.

(3) Hybridoma selection and cloning

The antibody titer of the hybridoma culture supernatant was determined by the EIA method described in Reference Example 5, using a UK-coupled microplate. At 10 to 20 days following fusion, hybridomas began to appear, along with an antibody that specifically binds to UK. The hybridomas showing particularly high affinity were subjected to cloning by the limiting dilution method.

The culture supernatant of the cloned hybridoma was subjected to screening in the same manner; those having high UK affinity were selected. As a result, UK1-3 [FERM BP-2083, IFO 50176] and UK1-87 [FERM BP-2084, IFO 50177] were obtained, both mouse hybridomas that produce an MoAb that specifically binds to UK. The immunoglobulin classes and subclasses of th antibodies produced by these hybridomas were identified by the Ouchterlony method as IgG_1 and IgG_{2b}, respectively.

Reference Exampl 9 Preparation of hybridoma that produces mouse anti-low-molecular-UK monoclonal antibody

(1) Immunization

Mice were immunized in the same manner as in Reference Example 8-(1) except that a commercially available two chain-low molecular UK (supplied by JCR Co.) was used in place of the UK described therein.

(2) Cell fusion

Cell fusion was carried out in accordance with the method described in Reference Example 8-(2).

(3) Hybridoma selection and cloning

Hybridoma screening was carried out by the EIA method, described in Reference Example 6, using a microplate to which low molecular UK was adsorbed; hybridomas that produced an anti-low-molecular-UK MoAb were obtained in the same manner as in Reference Example 8-(3) . Out of them, mouse hybridoma UK1-6 [IFO 50208, FERM BP-2548], a hybridoma that produces an anti-low-molecular-UK MoAb capable of specifically binding to UK without degrading the fibrinolytic capability thereof, was obtained. The immunoglobulin class and subclass of the antibody UK 1-6, produced by the hybridoma thus obtained, was identified as IgG_1 (κ chain) by the Ouchterlony method.

Reference Example 10 EIA for anti-glucuronidase antibody assay

An antigen-sensitized plate was prepared in the same manner as in Reference Example 5 except that β-glucuronidase (produced by Sigma Co.) was used in place of the UK described therein. EIA was then carried out in the same manner as in Reference Example 5, to determine the anti-glucuronidase antibody titer.

Reference Example 11 Glucuronidase enzyme reaction neutralization experiment

An antibody-sensitized plate was prepared in the same manner as in Reference Exampl 5 except that an anti-mouse-IgG antibody was used in

place of the UK described therein. The subject mouse anti-glucuronidase antibody solution was then added, followed by reaction at room temperature for 2 hours. After plate washing with PBS-Tw, a 25 μg/mℓ β-glucuronidase solution was added, followed by reaction at room temperature for 2 hours. After the plate was thoroughly washed, 1.0 mM synthetic substrate p-nitrophenyl-β-D-glucuronide in solution in 0.14 M acetate buffer (pH 4.6) containing 0.14% Triton X-100 was added, followed by enzyme reaction at 37°C for 40 minutes. After terminating the reaction by the addition of 2.5 M 2-amino-2-methyl-1,3-propanediol, the amount of pigment formed was measured at 415 nm using Multiscan.

Reference Example 12 Preparation of anti-hTfR-antibody-producing hybridoma

(1) Purification of hTfR

1.5 kg of human placenta tissue was cut into small pieces and blended in PBS (pH 7.5), followed by centrifugation. The resulting sediment was homogenized in PBS containing 4% Triton X-100. This homogenate was ultrasonicated and then centrifuged. To the resulting supernatant was added ammonium sulfate at about 32 g per 100 mℓ supernatant. After salting-out, this mixture was applied to a column coupled with anti-hTf antibody, followed by thorough washing with 20mM disodium phosphate buffer (hereinafter also referred to as PB), pH 7.5, containing 0.5 M NaCl. The hTfR fraction eluted with a 0.02 M glycine buffer solution (pH 10.0) containing 0.5 M NaCl and 0.5% Triton X-100 was applied to an hTf-coupled column. After the column was washed with PB containing 1 M NaCl, elution was conducted using a 0.05 M glycine buffer solution (pH 10.0) containing 1 M NaCl and 1% Triton X-100 to yield about 1.5 mg of a purified sample of hTfR.

(2) Immunization

To a 200 μg/mℓ solution of the above purified sample of hTfR in physiological saline was added an equal volume of Freund's complete adjuvant, followed by thorough emulsification. The resulting emulsion was then administered intraperitoneally and subcutaneously at the back to BALB/c mice (female, n=10, 20 μg/mℓ/mouse). Additional immunization was conducted at intervals of 3 weeks. The animal that showed the maximum serum antib dy titer 2 w ks after 4 additional immunizations was

intravenously given the same hTfR antigen solution as specified above (30 μg/0.1 mℓ physiological saline/mouse).

(3) Cell fusion

3 days after the final immunization, the spleen was excised and a splenocyte suspension was prepared by a conventional method (approximately 10^8 cells). To this suspension was added 2×10^7 mouse myeloma cells (P3U1), followed by cell fusion in accordance with the method described in Reference Example 8-(2). After selection of parent cells in HAT medium, cultivation was continued using HT medium which had the same composition as that of HAT medium, but not including aminopterin.

(4) Selection and cloning of hybridomas

A commercially available anti-mouse IgG rabbit antibody solution (20 μg/mℓ) was dispensed to a 96-well microplate at 100 μℓ per well. After this microplate was allowed to stand at 4°C overnight, PBS (pH 7.3) containing 2% BSA was added to prepare a sensitized plate. The purified sample of hTfR obtained in (1), after being labeled with HRP in accordance with a conventional method, was used for EIA [T. Kitagawa: *Yuki Gosei Kagaku*, *42*, 283 (1984)]. Accordingly, the culture supernatant of hybridomas was added to the above second antibody-sensitized plate, and reaction was carried out at room temperature for 2 hours. After the plate was washed with PBS, HRP-labeled hTfR was added, followed by reaction at room temperature for 2 hours. Enzyme reaction was then carried out by the method described in Reference Example 3, to determine the antibody titer.

The hybridoma showing especially high binding activity was subjected to cloning by limiting dilution method to yield anti-hTfR-antibody-producing hybridoma 22C6. The present antibody was identified as the IgG_1 (κ chain) subclass, exhibiting high affinity to human leukemia cell strain K562 and human epidermoid carcinoma cell line A431.

Example 1 Preparation of hybridoma that produces anti-glucuronidase monoclonal antibody

(1) Immunization

To a 500 μg/mℓ solution of a commercially available β-glucuronidase in saline, an equal amount of Freund's complete adjuvant was added; this mixture was thoroughly emulsified. The resulting emulsion was intraperitoneally and subcutaneously (at the back) administered to BALB/c mice (female, 50 μg/0.2 mℓ/mounse), followed by booster immunization at intervals of 2 to 3 weeks. The animals showing maximum serum antibody titer at 10 days after the third booster immunization received intravenous administration of a β-glucuronidase solution (100 μg/0.2 mℓ saline/mounse).

(2) Cell fusion

Spleens were excised from mice that showed high serum antibody titer, as determined by the EIA method described in Reference Example 10; cell fusion was carried out in accordance with the method described in Reference Example 8-(2).

(3) Hybridoma selection and cloning

Fused cells that appeared at 10 to 20 days following fusion were screened by the EIA method described in Reference Example 10; the hybridomas showing particularly high affinity were subjected to cloning by the limited dilution method.

The cloned hybridomas were selected in the same EIA method to yield BG1-5 [FERM BP-2688, IFO 50219], a mouse hybridoma that produces an MoAb that specifically binds to glucuronidase. The antibody produced by this hybridoma was identified as IgG_1. The neutralization experiment described in Reference Example 11 revealed that this antibody does not neutralize glucuronidase enzyme activity.

Example 2 Preparation of hybrid hybridoma that produces anti-human-cancer-cell-anti-UK bispecific antibody

(1) Cell fusion

Hybridoma RCS-1, which produces an anti-human-renal-cell-cancer MoAb, obtained in Reference Example 4, and hybridoma UK1-6, which produces an anti-UK MoAb, obtained in Reference Example 9, were each incubated in Iskove-Ham F-12 mixed medium containing 0.5 μg/mℓ FITC and 1.5 μg/mℓ TRITC at 37°C for 30 minutes for fluorescent staining. An LSM solution (commercially available from Wako Pure Chemical Industries Ltd.) was then added, and the dead cells were removed; the two hybridomas were then mixed at a ratio of 1 to 1 for cell fusion using PEG 6000 by the method described in Reference Example 8-(2).

After incubation at 37°C for 2 hours, the cell mixture was applied to FACS, and 25000 fluorescein-rhodamine double stained cells were separated and seeded, at 10 cells per well, to a 96-well microplate seeded with 5×10^5 cells/well mouse thymocytes as feeders, and cultivated.

(2) Hybrid hybridoma selection and cloning

The culture supernatant from each well in which cell growth occurred 1 to 2 weeks after fusion was subjected to Cell-EIA to determine the bispecific antibody titer. Specifically, to the microplate coupled with renal cancer cell AM-RC-7, prepared in Reference Example 3, the subject hybrid hybridoma culture supernatant was added, followed by reaction at room temperature for 2 hours. After plate washing with 0.2% BSA medium, biotin-labeled UK was added, followed by reaction at room temperature for 2 hours. After HRP-labeled avidin reaction at room temperature for 1 hour, the plate was washed and the enzyme activity bonded to the solid phase was determined by the method described in Reference Example 3.

The cells in wells showing high bispecific antibody titer were subjected to cloning by the limiting dilution method, yielding the desired bispecific-antibody-producing tetraoma.

(3) Purification of bispecific antibody

To BALB/c mice pretreated by intraperitoneal administration of 0.5 mℓ mineral oil, mouse hybrid hybridomas (tetraomas) were inoculated intraperitoneally at 5×10^6/mouse. Ascites fluid, whose retention occurred about 10 to 20 days after inoculation, was collected and subjected to salting-out with 50% saturated ammonium sulfate to yield an IgG fraction. After dialysis with 20 mM PBS (pH 7.5), th IgG fraction was applied to a UK-

coupled Cellulofin column, followed by elution with 0.2 M glycine-HCl buffer at pH 2.9. After dialysis with PBS, the acid-eluted fraction was applied to a hydroxyapatite column to purify the desired bispecific anti-human-cancer-cell-anti-UK antibody.

Example 3 Synthesis of tripeptidated drug 1

(1) Synthesis of Gly-Arg-OMe

To a solution of 4.6 g of carbobenzyloxyglycine (ZGly) in 20 mℓ of dimethylformamide (DMF), 4.34 g of N-hydroxy-5-norbornene-2,3-dicarboxyimide (HONB) and 4.9 g of dicyclohexylcarbodiimide (DCC) were added with ice cooling, followed by stirring for 3.5 hours. Then, 0.7 g more of HONB and 0.8 g more of DCC were added, followed by stirring for 2 hours. After the reaction mixture was filtered, the resulting filtrate, along with 5.22 g of arginine methyl ester (Arg-OMe) hydrochloride, was added to a solution of 2.8 mℓ of triethylamine in 30 mℓ DMF with ice cooling. After stirring at room temperature for 2 hours, the mixture was kept standing overnight. After precipitate removal by filtration, the filtrate was concentrated under reduced pressure. The resulting residue was diluted with water and washed with ethyl acetate. The water layer was concentrated under reduced pressure to yield a colorless oily substance (9.65 g). This oily substance was dissolved in 170 mℓ of methanol (MeOH) containing 20 mℓ of 1 N HCℓ, followed by catalytic reduction in a hydrogen stream in the presence of palladium black (500 mg). After stirring in the hydrogen stream for 4.5 hours, the catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure. The resulting residue was stored in DMF (30 mℓ) solution for use for the next reaction.

(2) Synthesis of Boc-Gly-Gly-Arg-OH

To a solution of 176 mg of t-butyloxycarbonylglycine (Boc-Gly) in 1 mℓ of DMF, 197 mg of HONB and 248 mg of DCC were added, followed by stirring with ice cooling. After precipitate removal by filtration, the filtrate was added to a mixture of a solution of the Gly-Arg-OMe obtained in (1) in DMF (2 mℓ) and triethylamine (187 μℓ) while stirring. After the resulting mixture was kept standing in a refrigerator overnight, the precipitate was removed by filtration. The residu obtained by concentrating the filtrate under reduced

pressure was subjected to column chromatography using silica gel (10 g), followed by elution with ethyl acetate-pyridine-acetic acid-water (60:20:6:10) to yield 210 mg of Boc-Gly-Gly-Arg-OMe. Then, 170 mg of the Boc-Gly-Gly-Arg-OMe was dissolved in 1 mℓ of a 1 N NaOH solution with ice cooling, and the reaction mixture was applied to cationic exchange resin (Biorex 70). The effluent fraction was collected and converted to HCℓ salt by the addition of 1 N HCℓ. The solvent was distilled off under reduced pressure to yield Boc-Gly-Gly-Arg-OH (148 mg).

NMR (D_2O) δ: 1.42 (9H, s, CH_3), 1.57-1.80 (4H, m, CH_2), 3.19 (2H, t, CH_2N), 3.81 (2H, s, CH_2CO), 3.95 (2H, s, CH_2CO), 4.13-4.30 (1H, m, N-CHCO)

MS m/z: 389 [M+H]+, 289 [M-Boc+2H]+

(3) Synthesis of Boc-Pro-Gly-Arg-OH

To a solution of 410 mg of t-butyloxycarbonylglycine (Boc-Pro) in 2 mℓ of DMF, 430 mg of HONB and 494 mg of DCC were added, followed by stirring with ice cooling. After precipitate removal by filtration, the filtrate was added to a mixture of a solution of the Gly-Arg-OMe obtained in (1) in DMF (4 mℓ) and triethylamine (374 μℓ) while stirring. After this mixture was kept standing overnight, the precipitate was removed by filtration. The residue obtained by concentrating the filtrate under reduced pressure was subjected to column chromatography using silica gel (20 g), followed by elution with ethyl acetate-pyridine-acetic acid-water (60:20:6:10) to yield 718 mg of Boc-Pro-Gly-Arg-OMe. Then, 398 mg of the Boc-Pro-Gly-Arg-OMe was dissolved in 2 mℓ of a 1 N NaOH solution with ice cooling, and the reaction mixture was applied to anionic exchange resin (AG-1 × 8). The effluent fraction was collected and applied to cationic exchange resin (Biorex 70). The effluent fraction was collected and converted to HCℓ salt by the addition of 1 N HCℓ. The solvent was distilled off under reduced pressure to yield Boc-Pro-Gly-Arg-OH (280 mg).

MS m/z: 429 [M+H]+, 329 [M-Boc+2H]+

(4) Synthesis of PyroGlu-Gly-Arg-OH

To a solution of 129 mg of pyroglutamic acid in 1 mℓ of DMF, 197 mg of HONB and 248 mg of DCC were added, followed by stirring with ice cooling. After precipitate removal by filtration, the filtrate was added to a mixture of a

Gly-Arg-OMe solution in DMF (2 mℓ) and triethylamine (187 µℓ), followed by the same treatment as (2) to yield 244 mg f PyroGlu-Gly-Arg-OMe. Then, 244 mg of the PyroGlu-Gly-Arg-OMe was dissolved in 1 mℓ of a 1 N NaOH solution with ice cooling. After stirring, the mixture was applied to cationic exchange resin (Biorex 70), and the effluent fraction was collected and converted to HCℓ salt by the addition of 1 N HCℓ. The solvent was distilled off under reduced pressure to yield PyroGlu-Gly-Arg-OH (200 mg).
MS m/z: 343 [M+H]+

(5) Synthesis of Boc-Gly-Gly-Arg-adriamycin

A solution of 28 mg of Boc-Gly-Gly-Arg-OH and 11 mg of 1-hydroxybenzotriazole (HOBT) in 0.3 mℓ of DMF was added to a solution of 6.4 mg of adriamycin (ADR) hydrochloride and 3 µℓ of N-ethylmorpholine in 0.1 mℓ of DMF, followed by stirring. After 3.8 mg of an aqueous solution of carbodiimide (WSC) was added to the reaction mixture described above, the solvent was distilled off under reduced pressure. To the residue, 0.2 mℓ of DMF, and then 8 mg of HONB, 9 µℓ of N-ethylmorpholine and 24 mg of WSC were added, followed by stirring at room temperature. After the reaction mixture was concentrated under reduced pressure, water was added to the residue. This residue dilution was added to a suspension of reversed phase silica gel (RP-8) in 5% CH_3CN-H_2O, followed by purification with an increasing density gradient of CH_3CN. Finally, elution was carried out using CH_3CN-H_2O-2M ammonium acetate (2:2:1). After the CH_3CN was distilled off, the eluted fraction was extracted with n-butanol. The resulting n-butanol extract was dried over sodium sulfate, and the solvent was distilled off under reduced pressure to yield Boc-Gly-Gly-Arg-ADR (3.55 mg).
MS m/z: 914 [M+H]+

(6) Synthesis of Boc-Pro-Gly-Arg-ADR

To a solution of 5 mg of ADR and 3 µℓ of N-ethylmorpholine in 0.5 mℓ of DMF, a solution of 18.1 mg of Boc-Pro-Gly-Arg-OH, 10 mg of HONB and 10 mg of WSC in DMF was added, followed by stirring. The mixture was then treated in the same manner as (5) to yield Boc-Pro-Gly-Arg-ADR.
MS m/z: 954 [M+H]+

(7) Synthesis fPyroGlu-Gly-Arg-ADR

To a solution of 5 mg of ADR and 3 μℓ of N-ethylmorpholine in 0.5 mℓ of DMF, a solution of 20.1 mg of PyroGlu-Gly-Arg-OH, 10 mg of HONB and 10 mg of WSC in DMF was added, followed by stirring. The mixture was then treated in the same manner as (5) to yield PyroGlu-Gly-Arg-ADR (0.14 g).
MS m/z: 867 [M+H]+

(8) Synthesis of Boc-Pro-Gly-Arg-TAN-1120

To a solution of 1.7 mg of TAN-1120, 4.2 mg of Boc-Pro-Gly-Arg-OH and 1.3 mg of HONB in 0.5 mℓ of DMF, 3.0 mg of WSC was added, followed by stirring at room temperature. The same treatment as (5) was followed to yield Boc-Pro-Gly-Arg-TAN-1120 from the fraction eluted with 40% CH_3CN-H_2O.
MS m/z: 1082 [M+H]+

(9) Synthesis of Boc-Gly-Gly-Arg-puromycin

To a solution of 6.2 mg of puromycin (PM)·2HCℓ and 6 μℓ of N-ethylmorpholine in 0.1 mℓ of DMF, 17.7 mg of Boc-Gly-Gly-Arg-OH, 7 mg of 1-hydroxybenzotriazole and 4 mg of water-soluble carbodiimide were added, followed by stirring at room temperature overnight. After the solvent was distilled off under reduced pressure, the residue was diluted with water and subjected to column chromatography using reversed phase silica gel (RP-8) for purification with an increased CH_3CN density gradient from 5% CH_3CN-H_2O. The fraction eluted with CH_3CN-H_2O-2M ammonium acetate (2:2:1) was collected. After the CH_3CN was distilled off under reduced pressure, n-butanol extraction was carried out. After the n-butanol extract was dried over anhydrous sodium sulfate, the solvent was distilled off under reduced pressure to yield Boc-Gly-Gly-Arg-PM (3.0 mg).
MS m/z: 842 [M+H]+, 742 [M-Boc+2H]+

Example 4 Cytotoxicity of tripeptidated drugs

The cytotoxicities of the three kinds of tripeptidated drugs obtained in Example 3 (Boc-Gly-Gly-Arg-ADR, Boc-Pro-Gly-Arg-TAN-1120 and Boc-Gly-Gly-Arg-PM, respectively) [Figs, 1 through 3; - ● -] on gastric cancer cell line NUGC4 or renal cancer cell line AM-RC-6 were compared with those of their activated bodies (ADR, TAN-1120 and PM, respectively) [Figs, 1 through 3; - ○ -]. Specifically, each drug was added at various concentrations to a microplate seeded with cultured human cancer cells at 5×10^3 cells/w ll,

followed by cultivation for 4 days. Then, in accordance with a known method, viable cells were counted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [H. Tada et al.: Jounal of Immunological Methods, *93*, 157 (1986)].

The results are shown in Figs. 1 through 3. ADR prodrug body [Fig. 1; - ● -] showed an about 1% activity relative to that of the activated body [Fig. 1; - ○ -] on NUGC4 or AM-RC-6 cancer cells. TAN-1120 prodrug body [Fig. 2; - ● -] showed a 1/10 to 1/100 or less cytotoxicity relative to that of the activated body [Fig. 2; - ○ -]. PM prodrug body [Fig. 3; - ● -] showed an about 1/4 activity relative to that of the activated body [Fig. 3; - ○ -].

Example 5 Tripeptidated drug activating reaction (1)

To a microplate seeded with 5×10^3/well renal cancer cell line AM-RC-6, the Boc-Gly-Gly-Arg-ADR or Boc-Gly-Gly-Arg-PM obtained in Example 3 was added, followed by the addition of trypsin to the Boc-Gly-Gly-Arg-ADR cell mixture, or UK to the Boc-Gly-Gly-Arg-PM cell mixture, and cultivation at 37°C. Three days later, viable cells were counted by the method described in Example 4, and prodrug activating reaction of trypsin or UK was measured.

The results are shown in Tables 1 and 2. Boc-Gly-Gly-Arg-ADR was decomposed by trypsin and showed significant increase in cytotoxicity. As well, Boc-Gly-Gly-Arg-PM was decomposed by UK and showed significant increase in cytotoxicity.

Table 1

Boc-Gly-Gly-Arg-ADR[1)]	% increase in cell count in the presence of trypsin (μg/mℓ)			
	0	1.0	10	100
2.0 μg/mℓ	100	67	45	32
4.0 μg/mℓ	100	64	54	28

1) Tripeptidated adriamycin

Table 2

Boc-Gly-Gly-Arg-PM[1)]	% increase in cell count in the presence of urokinase(μg/mℓ)		
	0	2.0	5.0
1.0 μg/mℓ	100	90	55
2.0 μg/mℓ	100	33	12

1) Tripeptidated puromycin

Example 6 Tripeptidated drug activating reaction (2)

The trypsin decomposition product of Boc-Gly-Gly-Arg-ADR and the UK decomposition product of Boc-Gly-Gly-Arg-PM obtained in Example 5 were subjected to reversed phase high performance liquid chromatography using an ODS column (YMC A-302 ODS 120A column, 4.6 × 150 mm, commercially available from YMC KK), and their chromatographic patterns were compared with those of their activated bodies ADR and PM. Elution was carried out with an eluent of 30% acetonitrile/0.01 M phosphate buffer (pH 3.0) at a flow rate of 1.0 mℓ/min; the ultraviolet absorbance of the column effluent was determined at 254 nm.

The results are shown in Figs. 4 and 5. Fig. 4 reveals that Boc-Gly-Gly-Arg-ADR (peak B; eluted at 10.2 minutes) was decomposed and activated into ADR (peak A; eluted at 3.6 minutes) by trypsin. Fig. 5 reveals that Boc-Gly-Gly-Arg-PM (peak B; eluted at 3.2 minutes) was decomposed and activated into PM (peak A; eluted at 1.8 minutes) by UK.

Example 7 Synthesis of prodrugs 2

(1) Synthesis of Boc-Gly-Gly-Arg-phenylenediamine mustard

Phenylenediamine mustard (PDM) was synthesized in accordance with a known method [W. C. J. Ross: Journal of Chemical Society, *183* (1949) and J. L. Everett et al.: Journal of Chemical Society, *1972* (1949)].

To a solution of 6.1 mg of PDM and 5 μℓ of N-ethylmorpholine in 0.3 mℓ of DMF was added, a DMF solution of 9.0 mg of Boc-Gly-Gly-Arg-OH, 7.7 mg of HOBT and 6 mg of WSC, followed by stirring. The mixture was then

treated in the same manner as in Example 3-(5) to yield Boc-Gly-Gly-Arg-PDM.

MS m/z: 603 [M+H]+, 503 [M-Boc+2H]+

(2) Synthesis of Boc-Gly-Gly-Arg-Val-ADR

A solution of 20 mg of ADR, 4 μℓ of N-ethylmorpholine, 28 mg of 3-nitro-2-pyridinesulfonyl-L-valine (Npys-Val), 12 mg of HOBT and 24 mg of WSC in 1 mℓ of DMF was stirred at room temperature. During the reaction, 4 μℓ of N-ethylmorpholine was added twice. After the solvent was distilled off under reduced pressure, extraction was carried out with ethyl acetate. The extract was washed with water and dried, and the solvent was distilled off under reduced pressure. The resulting residue was purified by column chromatography using silica gel (3 g). The 2% methanol-chloroform eluted fraction was concentrated under reduced pressure to yield Npys-Val-ADR as an orange-red crystal (27 mg). To a solution of 13.1 mg of the Npys-Val-ADR in 1 mℓ of dioxane, 0.1 mℓ of a 1 N aqueous hydrochloric acid was added, followed by stirring at room temperature for 40 minutes. Aqueous sodium bicarbonate was then added to neutralize the solution, followed by n-butanol extraction. After the extract was washed with water and dried, the solvent was distilled off under reduced pressure. The resulting residue was purified by column chromatography using silica gel (3 g) to yield Val-ADR (7 mg) as an orange-red oily substance from the 10% methanol-chloroform eluted fraction.

MS m/z: 643 [M+H]+

To a DMF solution of 2.0 mg of Val-ADR, 7.1 mg of Boc-Gly-Gly-Arg-OH and 6.9 mg of HOBT, 7.0 mg of WSC was added, followed by stirring at room temperature. The solvent was distilled off under reduced pressure and the resulting residue was treated in the same manner as in Example 3-(5) to yield Boc-Gly-Gly-Arg-Val-ADR.

MS m/z: 1035 [M+Na]+

(3) Synthesis of QS4-Gly-Gly-Arg-PM

To a suspension of 628 mg of glycine ethyl ester (Gly-OEt) in 5 mℓ of DMF, 630 μℓ of N-ethylmorpholine was added, followed by stirring at room temperature. A solution of 1.178 g of 6-(3-carboxypropyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone (QS-4) in 3 mℓ of DMF was then added, followed by

the addition of 955 mg of WSC and stirring at room temperature overnight. After the solvent was distilled off under reduced pressure, the residue was diluted with water and extracted with ethyl acetate. After the extract was washed with water and dried, the solvent was distilled off under reduced pressure. The resulting residue was purified by column chromatography using silica gel (48 g). The 1% methanol-chloroform eluted fraction was concentrated under reduced pressure and the residue was crystallized from ethyl acetate-n-hexane to yield 4-(2,3-dimethoxy-5-methyl-1,4-benzoquinone-6-yl)butanolglycine ethyl ester (QS-4-Gly-OEt, 561 mg) in the form of an orange-yellow needle. An ethyl acetate solution of 94 mg of the QS-4-Gly-OEt was converted to a hydroquinone by mixing in an aqueous solution of hydrosulfite while shaking; the ethyl acetate was then distilled off under reduced pressure. After the residue was dissolved in MeOH, 1 N NaOH was added and hydrolysis was carried out in an N_2 gas stream. The hydrolyzate was acidated with 1 N HCℓ and extracted with ethyl acetate. After the extract was washed with water and dried, the solvent was distilled off under reduced pressure. The resulting residue was dissolved in DMF (0.2 mℓ), followed by addition of Gly-Arg-OMe (141 mg), HOBT (39 mg) and WSC (110 mg) and stirring at room temperature overnight. HOBT (39 mg) and WSC (55 mg) were further added, followed by stirring. After the solvent was distilled off under reduced pressure, water was added, and the unreacted substance was extracted with ether. The residue obtained by concentrating the aqueous solution under reduced pressure was subjected to column chromatography using silica gel (5 g), followed by elution with ethyl acetate-pyridine-acetic acid-water (60:20:6:10) to yield QS-4-Gly-Gly-Arg-OMe. To an aqueous solution of the obtained QS-4-Gly-Gly-Arg-OMe, an aqueous solution of hydrosulfite was added, followed by stirring. Then, 1 N NaOH was added, and hydrolysis was carried out. After neutralization with 1 N HCℓ, the hydrolyzate was applied to cationic exchange resin (Biorex 70). The effluent fraction and the 1 N pyridine eluted fraction were combined, and the solvent was distilled off under reduced pressure to yield QS-4-Gly-Gly-Arg (80 mg). To a DMF solution of 10 mg of puromycin hydrochloride (PM·2HCℓ) and 10 μℓ of N-ethylmorpholine, 20 mg of the aforementioned QS-4-Gly-Gly-Arg, 7 mg of HOBT and 4.5 mg of WSC were added, followed by stirring at room temperature overnight. After the solvent was distilled off under reduced pressure, th resulting residu was dissolved in MeOH and xidized to a

quinone body with an aqueous solution of ferric chloride. The solvent was then distilled off under reduced pressure. The residue was treated in the same manner as in Example 3-(5) to yield QS-4-Gly-Gly-Arg-PM in the form of a yellow oily substance.

MS m/z: 994 $[M+3H]^+$

(4) Synthesis of QS-10-Gly-Gly-Arg-PM

The starting material 6-(9-carboxynonyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone (QS-10) was treated in the same manner as in (3) above and crystallized from ethyl acetate-n-hexane to yield 10-(2,3-dimethoxy-5-methyl-1,4-methyl-benzoquinone-6-yl)decanoylglycine ethyl ester (QS-10-Gly-OEt) in the form of an orange-yellow needle (mp. 77 to 77.5°C).

NMR ($CDC\ell_3$) δ: 1.23-1.40 (15H, m, CH_3, CH_2), 1.63 (2H, m, CH_2), 2.01 (3H, s, nuclear CH_3), 2.24 (3H, t, CH_2CON), 2.44 (2H, t, nuclear CH_2), 3.99 (6H, s, OCH_3), 4.03 (2H, d, NCH_2COO), 4.22 (2H, q, OCH_2)

After hydrolysis, QS-10-Gly-OEt was condensed with the dipeptide Gly-Arg-OMe to yield QS-10-Gly-Gly-Arg-OMe in the form of a yellow oily substance.

MS m/z: 639 $[M+3H]^+$

After hydrolysis, QS-10-Gly-Gly-Arg-OMe was condensed with PM in the presence of WSC to yield QS-10-Gly-Gly-Arg-PM in the form of a yellow oily substance.

MS m/z: 1076 $[M+H]^+$

(5) Synthesis of Bz-Gly-Ala-Pro-Gly-Arg-PM

To a solution of 70 mg of Bz-Gly-Ala-Pro (Peptide Kenkyukai) in 1 mℓ of DMF, 50 mg of HONB and 58 mg of DCC were added, followed by stirring at room temperature for 2 hours. After this reaction mixture was filtered and the precipitate was removed, the filtrate was added to a solution of 30 μℓ of triethylamine and 112 mg of Gly-Arg-OMe hydrochloride in 1 mℓ of DMF, followed by stirring at room temperature overnight. After the precipitate was removed by filtration, the filtrate was concentrated under reduced pressure. The resulting residue was subjected to column chromatography using silica gel (6 g) for elution with ethyl acetate-pyridine-acetic acid-water (60:20:6:10) to yield Bz-Gly-Ala-Pro-Gly-Arg-OMe. To an aqueous soluti n of the obtained Bz-Gly-Ala-Pro-Gly-Arg-OMe, 1 N NaOH was added, followed by stirring at

room temperature. Th reaction mixture was applied to cationic exchange resin (Biorex 70). The effluent fraction and th 1 N pyridin eluted fraction were combined, and the solvent was distilled off under reduced pressure to yield Bz-Gly-Ala-Pro-Gly-Arg (70 mg) in the form of a colorless oily substance. To a solution of 10.3 mg of PM and 10 μℓ of N-ethylmorpholine in 300 μℓ of DMF, a solution of 20 mg of the above-mentioned Bz-Gly-Ala-Pro-Gly-Arg in 300 μℓ of DMF and 7 mg of HOBT was added, followed by the addition of 9.0 mg of WSC and stirring at room temperature for 4 hours. After the mixture was kept standing in a cold room overnight, the solvent was distilled off under reduced pressure, and the residue was treated in the same manner as in Example 3-(5) to yield Bz-Gly-Ala-Pro-Gly-Arg-PM in the form of a colorless oily substance.

MS m/z: 1014 [M+H+]

(6) Synthesis of Z-Gly-Pro-Leu-Gly-Gly-Arg-PM

The starting material commercial Z-Gly-Pro-Leu-Gly was treated in the same manner as in (5) above to yield Z-Gly-Pro-Leu-Gly-Gly-Arg-PM in the form of a colorless oily substance.

MS m/z: 1143 [M+H+]

Example 8 Prodrug activating reaction (3)

To a microplate seeded with human epidermoid carcinoma cell line A431 at 7×10^3 cell/well, the Boc-Gly-Gly-Arg-PDM, Boc-Gly-Gly-Arg-Val-ADR, QS-4-Gly-Gly-Arg-PM, QS-10--Gly-Gly-Arg-PM and Bz-Gly-Ala-Pro-Gly-Arg-PM obtained in Example 7 were added, followed by addition of UK and cultivation at 37°C. The prodrug activating reaction of UK was then determined by the method described in Example 5.

The results are shown in Table 3. All prodrug bodies were activated by UK and showed strong cytotoxicity.

Table 3

Prodrug body	UK concentration (μg/mℓ)	% cell growth
Boc-Gly-Gly-Arg-PDM 1.0 μg/mℓ	0	100
	1	87
	4	66
Boc-Gly-Gly-Arg-Val-ADR 5.0 μg/mℓ	0	100
	1	92
	5	50
QS-4-Gly-Gly-Arg-PM 1.0 μg/mℓ	0	100
	1	84
	4	64
QS-10-Gly-Gly-Arg-PM 2.0 μg/mℓ	0	100
	2	86
	8	56
Bz-Gly-Ala-Pro-Gly-Arg-PM 1.0 μg/mℓ	0	100
	2	93
	8	73

Example 9 Preparation of hybrid hybridoma that produces anti-hTfR-anti-UK bispecific antibody

(1) Cell fusion

Hybridoma 22C6, which produces an anti-hTfR MoAb, obtained in Reference Example 12, and hybridoma UK1-6, which produces an anti-UK MoAb, obtained in Reference Example 9, were each incubated in Iskove-Ham F-12 mixed medium containing 0.5 μg/mℓ FITC and 1.5 μg/mℓ TRITC at 37°C for 30 minutes for fluorescent staining. An LSM solution (commercially available from Wako Pure Chemical Industries Ltd.) was then added, and the dead cells were removed; the two hybridomas were then mixed at a ratio of 1 to 1 for cell fusion using PEG 6000 by the method described in Reference Example 8-(2).

After incubation at 37°C for 2 hours, the cell mixture was applied to FACS, and 25000 fluorescein-rhodamine double stained cells were separated

and seeded, at 10 cells per well, to a 96-well microplate seeded with 5×10^5 cells/well mouse thymocytes as feeders, and cultivated.

(2) Hybrid hybridoma selection and cloning

The culture supernatant from each well in which cell growth occurred 1 to 2 weeks after fusion was subjected to Cell-EIA to determine the bispecific antibody titer. Specifically, to the microplate coupled with human cancer cell A431, prepared in Reference Example 3, the subject hybrid hybridoma culture supernatant was added, followed by reaction at room temperature for 2 hours. After plate washing with 0.2% BSA medium, biotin-labeled UK was added, followed by reaction at room temperature for 2 hours. After HRP-labeled avidin reaction at room temperature for 1 hour, the plate was washed and the enzyme activity bound to the solid phase was determined by the method described in Reference Example 3.

The cells in wells showing high bispecific antibody titer were subjected to cloning by the limiting dilution method, the desired bispecific-antibody-producing mouse tetraoma UTF 20-7 was obtained.

The result is shown in Figure 6.

(3) Purification of bispecific antibody

To BALB/c mice pretreated by intraperitoneal administration of 0.5 mℓ mineral oil, mouse hybrid hybridomas (tetraomas) were inoculated intraperitoneally at 5×10^6/mouse. Ascites fluid, whose retention occurred about 10 to 20 days after inoculation, was collected and subjected to salting-out with 50% saturated ammonium sulfate to yield an IgG fraction. After dialysis with 20 mM PBS (pH 7.5), the IgG fraction was applied to a UK-coupled Cellulofine column, followed by elution with 0.2 M glycine-HCl buffer at pH 2.9. After dialysis with PBS, the acid-eluted fraction was applied to a hydroxyapatite column, the desired bispecific anti-hTfR-anti-UK antibody was purified.

By the present method, about 8.2 mg of the desired bispecific antibody UTF 20-7 was obtained.

Example 10 Prodrug activating reaction by bispecific antibody

To a microplate seeded with 1.0×10^4 cell/well of human pidermoid carcin ma cell lin A431 and mous leukemia c ll line P388, the

immunocomplex comprising the purified bispecific antibody obtained in Exampl 9 and UK (1:1) was added, foll wed by reaction at 5°C for 30 minutes. After cells were washed at a low temperature, the prodrug Boc-Gly-Gly-Arg-Val-ADR, described in Example 7-(2) or the prodrug QS-10-Gly-Gly-Arg-PM described in Example 7-(4) was added at a final concentration of 5.0 µg/ml and 0.2 µg/ml, respectively. The prodrug activating reaction of the immunocomplex comprising UK and the bispecific antibody, bound to cell surface, was then determined by the method described in Example 5. The results are shown in Table 4. All prodrugs were activated by the immunocomplex comprising UK and the bispecific antibody and showed strong cytotoxicity against the target cell line A431. On the other hand, they showed no cytotoxicity against the non-target cell line P388.

Table 4

prodrug body	immnocomplex concentration (µg/ml as UK)	% cell growth	
		A431	P388
Boc-Gly-Gly-Arg-Val-ADR (0.5 µg/mℓ)	0	100	100
	3	75	103
	10	58	95
QS-10-Gly-Gly-Arg-PM (2.0 µg/mℓ)	0	100	100
	3	84	98
	10	62	95

CLAIMS

1. A bispecific hybrid monoclonal antibody having specificities against a human cancer cell and a prodrug-activating enzyme.

2. An antibody as claimed in claim 1, wherein said prodrug-activating enzyme is a protease.

3. An antibody as claimed in claim 2, wherein said protease is urokinase.

4. An antibody as claimed in claim 1, wherein said prodrug-activating enzyme is a glycosidase.

5. An antibody as claimed in claim 4, wherein said glycosidase is glucuronidase.

6. An antibody as claimed in claim 1, wherein said prodrug-activating enzyme is an enzyme which converts an inactive anticancer prodrug into active anticancer agent.

7. An antibody as claimed in claim 6, wherein said prodrug is a peptidated anticancer agent.

8. An antibody as claimed in claim 6, wherein said prodrug is a tripeptidated anticancer agent.

9. An antibody as claimed in claim 6, wherein said prodrug is a glucuronidated anticancer agent.

10. An antibody as claimed in claim 6, wherein said anticancer agent is an anticancer agent selected from the group consisting of adriamycin, cisplatin, melphalan, methotrexate, mitomycin C, vincristine, puromycin, phenylenediamine mustard, ansamitocins, TAN-1120 and related compounds thereof.

11. An antibody as claimed in claim 6, wherein said anticancer agent is an anticancer agent selected from the group consisting of adriamycin, puromycin, phenylenediamine mustard, ansamitocins and TAN-1120.

12. A polydoma that produces an antibody as claimed in claim 1.

13. A tetraoma wherein said tetraoma is the fusion product of a hybridoma that produces an anti-human-cancer antibody and a hybridoma that produces an anti-urokinase antibody and wherein said tetraoma produces a bispecific hybrid monoclonal antibody having specific binding affinities to both a human cancer cell and urokinase.

14. A tetraoma as claimed in claim 13, wherein said anti-human-cancer antibody producing hybridoma is an anti-human-cancer-cell-membrane-surface-antigen antibody producing hybridoma.

15. A tetraoma as claimed in claim 13, wherein said anti-human-cancer antibody producing hybridoma is an anti-human-transferrin-receptor antibody producing hybridoma.

16. A tetraoma as claimed in claim 13, wherein said anti-human-cancer antibody producing hybridoma is Mouse hybridoma 22C6.

17. A tetraoma as claimed in claim 13, wherein said anti-urokinase antibody producing hybridoma is Mouse hybridoma UK 1-6.

18. The Mouse tetraoma UTF20-7.

19. An anti-human-cancer-protein complex comprising an antibody as claimed in claim 1 and a prodrug activating enzyme immunologically coupled thereto.

20. A method for producing a polydoma which produces a bispecific hybrid monoclonal antibody whose two specificities are respectively against a human cancer cell and a prodrug-activating enzyme, which comprises fusing an anti-human-cancer-cell-antibody-producing hybridoma or cell and a hybridoma or cell which produces an antibody against prodrug-activating enzyme.

21. The method as claimed in claim 20, wherein said prodrug-activating anzyme is protease.

22. The method as claimed in claim 21, wherein said protease is urokinase.

23. The method as claimed in claim 20, wherein said prodrug-activating enzyme is glycosidase.

24. The method as claimed in claim 23, wherein said glycosidase is glucuronidase.

25. The method as claimed in claim 20, wherein said polydoma is a tetraoma which is obtained by fusing an anti-human-cancer-cell-antibody-producing hybridoma and an anti-prodrug-activating-enzyme-antibody-producing hybridoma and which produces a bispecific hybrid monoclonal antibody having binding affinities to both a human cancer cell and a prodrug-activating-enzyme.

26. The method as claimed in claim 20, wherein said polydoma is a tetraoma which is obtained by fusing an anti-human-cancer-cell-antibody-

producing hybridoma and an anti-urokinase-antibody-producing hybridoma and which produces a bispecific hybrid monoclonal antibody having binding affinities to both a human cancer cell and urokinase.

27. A method for producing a bispecific hybrid monoclonal antibody having binding affinities against a human cancer cell and a prodrug-activating enzyme, which comprises cultivating the polydoma as claimed in claim 12 in a liquid medium or a peritoneal cavity of animal, and harvesting said antibody from culture supernatant or ascites fluid.

28. The method as claimed in claim 27, wherein said prodrug-activating enzyme is protease.

29. The method as claimed in claim 28, wherein said protease is urokinase.

30. The method as claimed in claim 27, wherein said prodrug-activating enzyme is glycosidase.

31. The method as claimed in claim 30, wherein said glycosidase is glucuronidase.

32. A method for therapy of cancer in a mammal, which comprises administering to said mammal an effective amount of the antibody as claimed in claim 1 in combination with an inactive anticancer prodrug.

33. A use of the antibody as claimed in claim 1 in combination with an inactive anticancer prodrug for therapy of cancer.

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MICROORGANISMS

Optional Sheet In connection with the microorganism referred to on page 3, line 25 of the description [1]

A. IDENTIFICATION OF DEPOSIT[2]

Further deposits are identified on an additional sheet ☐[3]

Name of depositary institution[4]
IFO: Institute for Fermentation, Osaka
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Address of depositary institution (including postal code and country)[4]
IFO: 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka-shi, Osaka 532 Japan
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Date of deposit [5]	Accession Number [6]
IFO: 26.08.88 FRI: 14.09.88	IFO- 50172 FERM BP- 2054

B. ADDITIONAL INDICATIONS[7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

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C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [3] (If the indications are not for all designated States)

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MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 3, line 28 of the description [1]

A. IDENTIFICATION OF DEPOSIT[2]

Further deposits are identified on an additional sheet ☐[3]

Name of depositary institution[4]
IFO: Institute for Fermentation, Osaka
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Address of depositary institution (including postal code and country)[4]
IFO: 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka-shi, Osaka 532 Japan
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MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 7, line 3 of the description [1]

A. IDENTIFICATION OF DEPOSIT[2]

Further deposits are identified on an additional sheet ☐[3]

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MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 12, line 12 of the description [1]

A. IDENTIFICATION OF DEPOSIT[2]

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MICROORGANISMS

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A. IDENTIFICATION OF DEPOSIT[2]

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MICROORGANISMS

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A. IDENTIFICATION OF DEPOSIT[2]

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MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 12, line 16 of the description [1]

A. IDENTIFICATION OF DEPOSIT[2]

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IFO: 13.12.89 FRI: 15.12.89	IFO- 50219 FERM BP- 2688

B. ADDITIONAL INDICATIONS[7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Ruel 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [3] (If the indications are not for all designated States)

States members of the European Patent Convention which have been designated for the purpose of a European Patent.

D. SEPARATE FURNISHING OF INDICATIONS[8] (leave blank if not applicable)

The indication listed below will be submitted to the International Bureau later[9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☑ The date of receipt (from the applicant) by the International Bureau[10]

was 12 March, 1991 (12.03.91) T. Shimomichi

(Authorized Officer)

Fig. 1

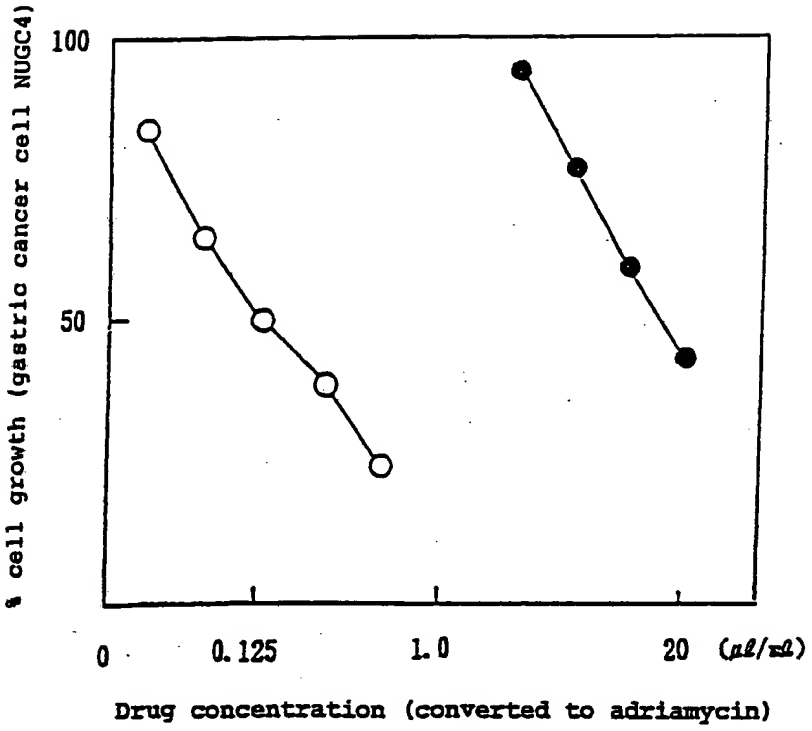

% cell growth (gastric cancer cell NUGC4)
100
50
0
0.125
1.0
20
(μℓ/mℓ)
Drug concentration (converted to adriamycin)

Fig. 2

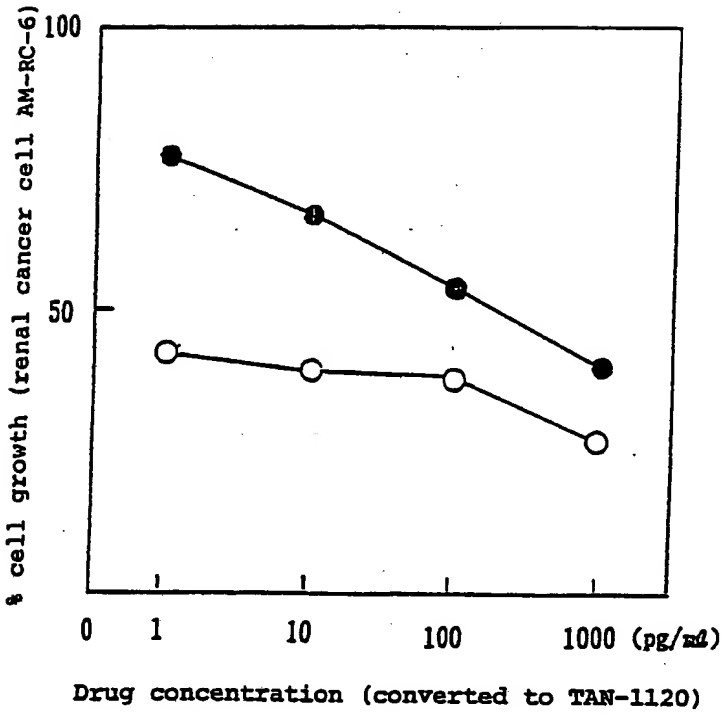

% cell growth (renal cancer cell AM-RC-6)
100
50
0
1
10
100
1000 (pg/ml)
Drug concentration (converted to TAN-1120)

Fig. 3

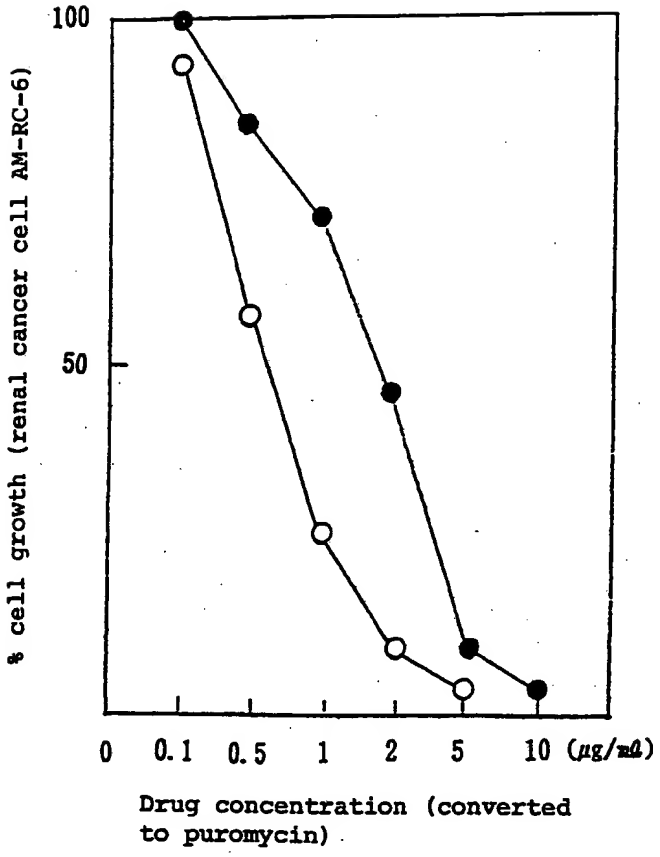

100
50
% cell growth (renal cancer cell AM-RC-6)
0
0.1
0.5
1
2
5
10 (μg/mℓ)
Drug concentration (converted to puromycin)

Fig. 4

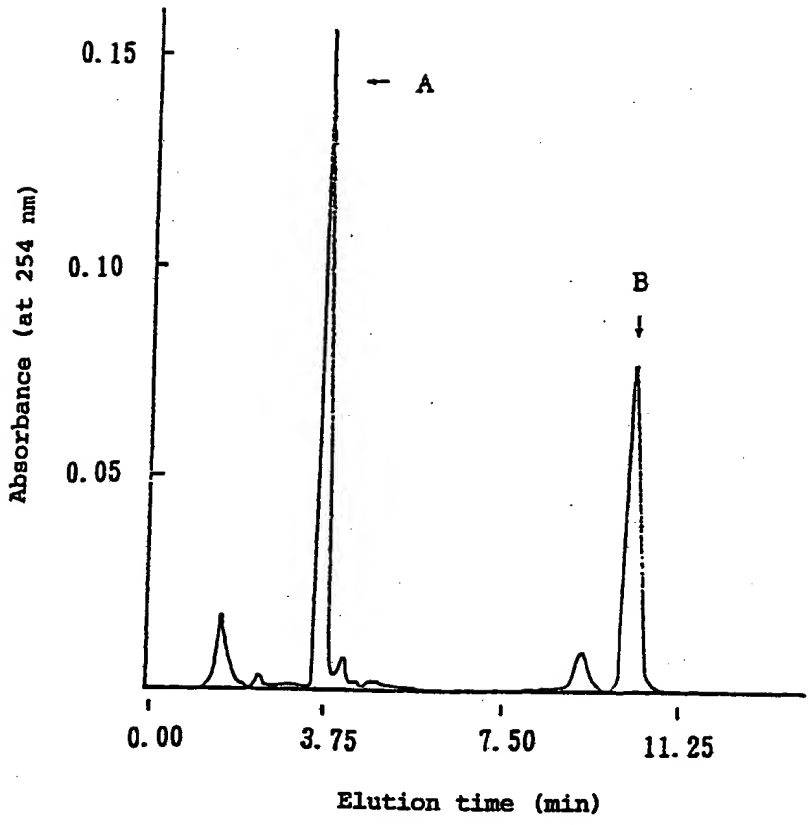

0.15
0.10
0.05
Absorbance (at 254 nm)
A
B
0.00
3.75
7.50
11.25
Elution time (min)

Fig. 5

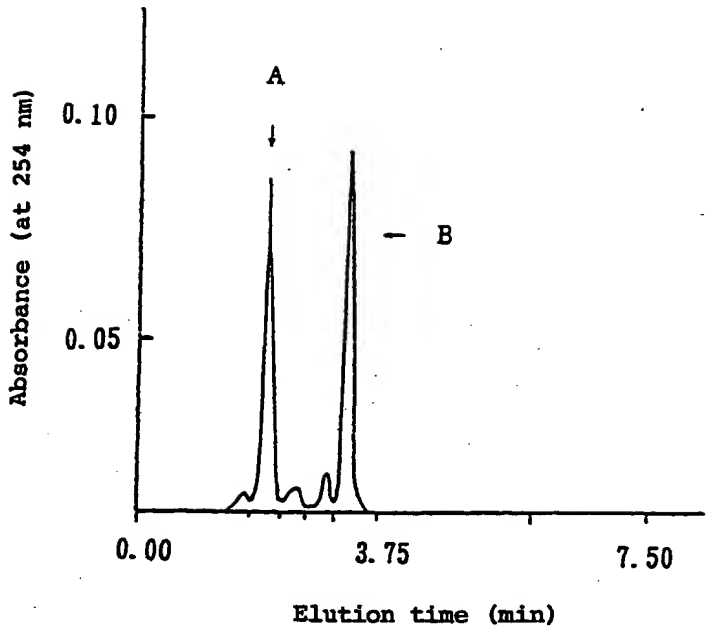
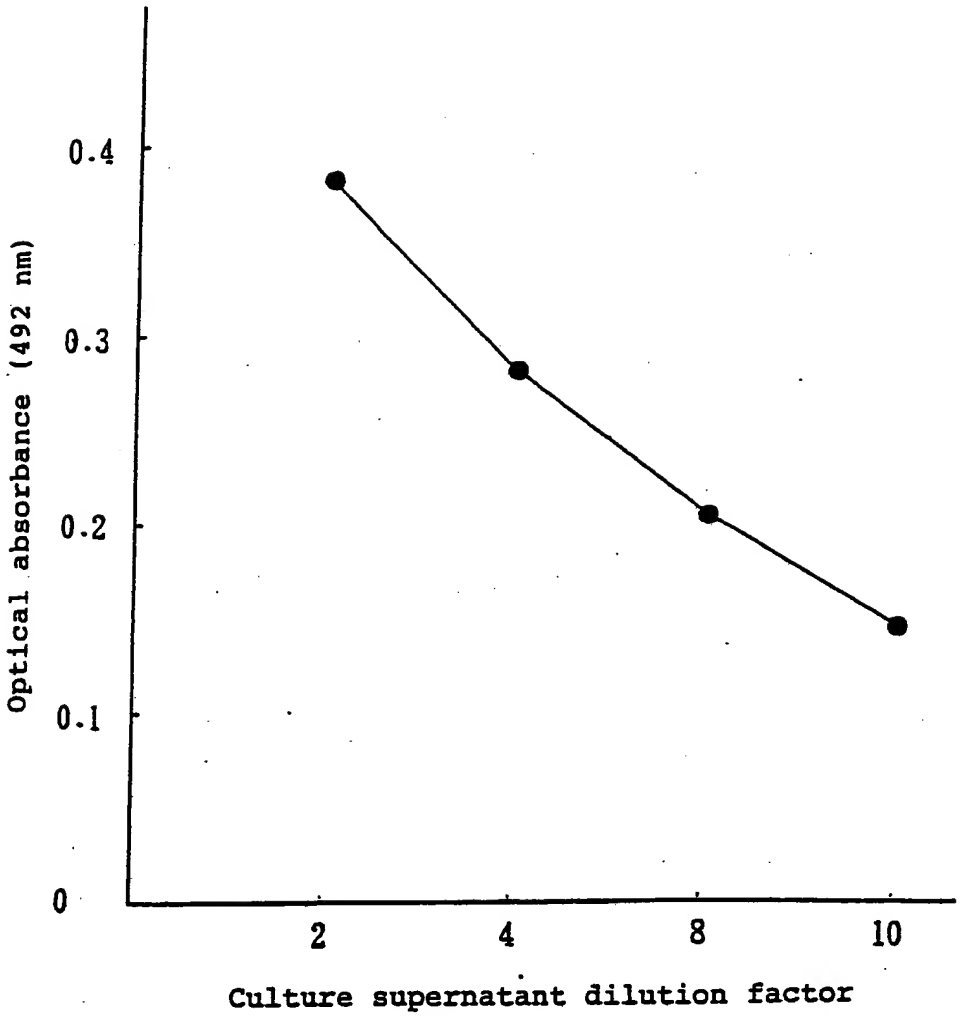


Fig. 6


Optical absorbance (492 nm)
0.4
0.3
0.2
0.1
0
2
4
8
10
Culture supernatant dilution factor

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP 90/01631

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 P 21/08, A 61 K 39/395, 47/48, C 12 N 5/12

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO, A1, 8910140 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED) 2 November 1989, see page 5, line 17 - page 6, line 6; page , line ; claims 6-8	1,2, 19
Y	WO, A1, 8807378 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LTD) 6 October 1988, see page 3, line 13 - page 4, line 27; page 6 - page 7, line 6; page 15 claims	1-14,19-31
Y	EP, A2, 0302473 (BRISTOL-MYERS COMPANY) 8 February 1989, see page 2 - page 4; page 7, line 37 - line 39	1-14,19-31

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14th March 1991	22.03.91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	p.p. MISS D. S. KOWALCZYK

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A2, 0142905 (BIO-RESPONSE INC.) 29 May 1985, see page 34, line 24 - page 36	1,19
A	Tibtech, Vol. 7, March 1989, Maria L. Fiani et al.: "Selective targeting of drugs ", see page 60 right column	1
A	WO, A1, 8703205 (CORAL SOCIEDADE BRASILEIRA DE PESQUISAS EDESENVOLVIMENTO LTDA) 4 June 1987, see the whole document	1,19
A	Chemical Abstracts, volume 109, no. 20, 14 November 1988, (Columbus, Ohio, US), Senter, Peter D. et al.: "Antitumor effects of antibody-alkaline phosphataseconjugates in combination with etoposide phosphate ", see page 369, abstract 176156p, & Proc. Natl. Acad. sci. 1988, 85(13), 4842-4846	1,19
A	Chemical Abstracts, volume 110, no. 14, 3 April 1989, (Columbus, Ohio, US), Bagshawe, K. D. et al.: "A cytotoxic agent can be generated selectively at cancer sites ", see page 426, abstract 121211t, & Br. J. Cancer 1988, 58(6), 700- 703	1,19
A	US, A, 4671958 (JOHN D. RODWELL ET AL.) 9 June 1987, see the whole document	1,19
A	WO, A1, 8601720 (CYTOGEN CORPORATION) 27 March 1986, see the whole document	1,19

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Tibtech, Vol. 6, February 1988, Gareth Williams: "Novel antibody reagents: production and potential ", see page 36 - page 37	1-14,19-31
Y	Bio/Technology, Vol. 5, March 1987, Arthur Klausner: "'Quadromas' yield bispecific antibodies ", see the whole document	1-14,19-31
A	Nature, Vol. 305, October 1983, C. Milstein et al.: "Hybrid hybridomas and their use in immunohistochemistry ",	19-31

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/JP 90/01631

SA 42625

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 31/01/91
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 8910140	02/11/89	NONE		
WO-A1- 8807378	06/10/88	EP-A-	0408546	23/01/91
EP-A2- 0302473	08/02/89	AU-D-	2020188	25/05/89
		JP-A-	2223532	05/09/90
		US-A-	4975278	04/12/90
EP-A2- 0142905	29/05/85	JP-A-	60072826	24/04/85
WO-A1- 8703205	04/06/87	EP-A-	0250477	07/01/88
US-A- 4671958	09/06/87	AU-B-	556446	06/11/86
		AU-D-	1199083	15/09/83
		CA-A-	1203164	15/04/86
		EP-A-	0088695	14/09/83
		JP-A-	58222035	23/12/83
WO-A1- 8601720	27/03/86	AU-B-	583854	11/05/89
		AU-D-	3016189	13/07/89
		AU-D-	4807185	08/04/86
		EP-A-	0175617	26/03/86
		JP-T-	62500175	22/01/87
		US-A-	4867973	19/09/89
		US-A-	4950738	21/08/90